Methanogonia costaricensis gen. nov. sp. nov., a novel methanogen from brackish water in Costa Rica.

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Abstract

The novel methanogenic strain CcM2M^T was isolated from brackish sediment of a river estuary in Cahuita National Park, Costa Rica. It displayed rod-shaped cells with angular cell ends, 0.4–0.7 µm in diameter, and 1.7–2.5 µm in length. The angular cell ends further indicate cell-cell connections that hint at an extraordinary, novel cell division strategy in this archaeon. Cells were non-motile, occurred as single cells, and stained Gram-negative. H_2/CO_2 was utilized for methanogenesis and acetate was required as carbon source. The optimal temperature for growth was 37°C (range: 20°C–40°C), with a doubling time of 10 h. Range and optima for pH was 5.0–6.5, and 0–3% for sodium chloride. Phylogenetically, the closest relatives were *Methanocellaceae* with 94.43% sequence identity on 16S rRNA and 78.54% on *mcrA* gene sequence level for *Methanocella arvoryzae*, and 93.79% sequence identity on 16S rRNA gene sequence level for *Methanooceanicella nereidis*. G+C content was 41.0 mol%. Considering all morphological, physiological, and phylogenetic aspects, CcM2M^T (= DSM 113456^T = JCM 39175^T) represents a novel genus, *Methanogonia* gen. nov., with the type strain *Methanogonia costaricensis* sp. nov.

Keywords

brackish water, methanogenesis, *Methanogonia*, *Methanocellaceae*, Costa Rica

Introduction

River estuaries are situated at the borderline of freshwater from terrestrial streams and saltwater from the oceans. In tropical climates, river outfalls are subject to temporal fluctuations, including variations in rainfall and humidity levels. With that, they constitute biologically challenging environments that require a certain adaptability of their residents. Even though the Caribbean is the historical cradle of mangroves (Rull 2023), the Costa Rican Caribbean coast only possesses patches of mangroves, mainly populated by the red mangrove (*Rhizophora mangle*). Its largest population is located at the national wildlife refuge Refugio Nacional de Vida Silvestre Gandoca-Manzanillo near the southeast Atlantic coast. For the Cahuita National Park, located 30 km further north, no classical mangrove species are reported (Cortés et al. 2010; Cortés 2016). Concerning their microbiology, mangroves, and estuary environments with brackish water influence have mostly been surveyed using molecular techniques. With that, the most prominent types of methanogens were detected to be hydrogenotrophic and methylotrophic species followed by acetoclastic methanogens (Banning et al. 2005; Zhang et al. 2020).

The order *Methanocellales* was described in 2008 and includes only four cultivated species at the time of writing (Sakai et al. 2008). It was recently assigned to the new class *Methanocellia* in the Genome Taxonomy Database (Chuvochina et al. 2023). All isolates belong to the family *Methanocellaceae* which inhabits so far only two genera, *Methanocella* and *Methanooceanicella*. Within the genus *Methanocella*, the validly published species *Methanocella paludicola* (Sakai et al. 2008), *Methanocella arvoryzae* (Sakai et al. 2010), and *Methanocella conradii* (Lü and Lu 2012) are implicated. The description of *M. paludicola* was the first successful cultivation and isolation of an organism from the Rice Cluster I (RC-I), a methanogenic lineage deeply branching within the orders *Methanosarcinales* and *Methanomicrobiales* (Conrad et al. 2006). Genetic evidence for this cluster was found worldwide, mainly in rice fields (Großkopf et al. 1998; Ramakrishnan et al. 2006; Wu et al. 2006), but also in river sediments (Kemnitz et al. 2004), peatland (Basiliko et al. 2003), and contaminated aquifers (Dojka et al. 1998). It is assumed that the organisms occupy a specific ecological niche and have thus been difficult to cultivate. This niche may include thriving under low hydrogen concentrations, acetate utilization as well as a certain aerotolerance (Conrad et al. 2006; Erkel et al. 2006). In brackish sediments, *Methanoregula* was reported to dominate the upper sediment (10 cm), while organisms of RC-I were more prominent in deeper regions (40 cm) (Galand et al. 2002). In subtropical estuarine marshes, *Methanocella* was highly underrepresented, whereas *Methanoregula*, *Methanobacterium*, *Methanolinea*, and *Methanosarcina* were abundant (Tong et al. 2017). A second representative within the *Methanocellaceae*, *Methanooceanicella nereidis* (Zhang et al. 2024), was recently isolated from the deep-sea, a potential methane hydrate bearing sediment offshore Taiwan. Its enrichment took five years, which again highlights the difficulty to cultivate members of the *Methanocellaceae*.

In this study, we report the isolation and characterization of a hydrogenotrophic methanogen, strain CcM2M^T, that clustered in the *Methanocellaceae* but showed significant phylogenetic, morphological, and physiological differences compared to all other published *Methanocella* species. Therefore, we propose the isolate CcM2M^T as type strain of a novel species within a novel genus, *Methanogonia costaricensis* gen. nov. sp. nov.

Material and Methods

Sampling, enrichment and isolation

The environmental samples derive from a mangrove-like sediment at a river estuary in the Cahuita National Park, Costa Rica. The area was exposed to freshwater from the river as well as salt water from the Caribbean Sea floating back into the river outfall (see supplementary material). Samples were taken using sterilized 100 mL glass bottles filled with N_2 and submerging them with a mixture of river sediment and brackish water. The bottles were closed with a rubber stopper and ultimately fixed with a screw cap. Samples were taken at approximately 70 cm below the water surface at 10–15 cm sediment depth in March 2019. Sediment temperature and pH were measured on-site, and samples were stored at 4°C upon further use. For enrichment, 0.5 mL of environmental sample was used. Cells were enriched and isolated on a sulfate-free minimal medium containing vitamins, SMS medium, with 0.1% acetate supplementation under H_2/CO_2 (80:20, v/v, 300 kPa) gas phase at 37°C as previously described (Dengler et al. 2023). Well-grown enrichment cultures were transferred twice under identical conditions. Then, cell isolation was carried out by two subsequent serial dilutions in 120 mL serum bottles with 20 mL medium in dilutions up to 10^{-10}. Purity was further checked using phase contrast light microscopy combined with tests for the typical fluorescence of methanogens during all physiological tests.

The isolate was cryo-preserved for long-term storage in the in-house culture collection (Bakterienbank Regensburg, University of Regensburg). 60 mL of well-grown culture was anaerobically centrifuged (3,000 g, 30 min) and the pellet was resuspended in fresh SMS medium containing 5% DMSO (final concentration; v/v). Ultimately, the concentrate was anaerobically transferred into glass capillaries, capillaries were sealed and stored above liquid nitrogen. After two weeks of cryo-storage, cells of one capillary were anaerobically transferred into a fresh inoculation medium and incubated under the described cultivation conditions to check for viability. For short-term storage (2–3 months), logarithmic cultures were stored at 4°C.

Morphological characterization and microscopic techniques

Gram-stain was performed as described previously (Boone and Whitman 1988) with *Escherichia coli* K12 (ATCC® 25922™) and *Bacillus atrophaeus* (ATCC® 9372™) as reference strains. Fluorescence and phase contrast light microscopy were performed as previously described (Dengler et al. 2022), and images were captured using a Canon EOS 60D camera (Canon Europa N.V., Amstelveen, The Netherlands) on an Olympus BX60 phase contrast light microscope (100x / 1.30 NA Oil). To survey motility under anaerobic conditions at defined temperatures, a temperature gradient-forming device was employed using a temperature gradient from 25–50°C in two-degree steps on an Olympus BX53 phase contrast light microscope (Mora et al. 2014).

For transmission electron microscopy (TEM), 1.5 mL of an exponentially grown culture were fixed with 1% glutardialdehyde (final concentration; v/v) for 10 min. The cells were then concentrated via centrifugation (10,000×g, 10 min), the supernatant was almost entirely removed, and the pellet was resuspended in the remaining excess medium (~ 20 µl). 10 µl of cell suspension was placed on a hydrophilized 400-mesh carbon-coated copper grid (Plano, Wetzlar, Germany). Samples were then shadowed with 1 nm Pt/C (15° angle; CFE 50 freeze-fracturing device; Cressington, Watford, England). In addition, freeze-etching of a native, unfixed cell pellet was performed as described (Rachel et al. 2002). Transmission electron micrographs were recorded using a JEM-

2100F field emission electron microscope (JEOL GmbH, Freising, Germany) operated at 200 kV equipped with an F416 CMOS camera (TVIPS, Gauting, Germany).

Identification of morphological characteristics including cell size, cell appendages, and S-layer was performed considering electron micrographs. All images were analyzed in ImageJ 1.54 (Schneider et al. 2012).

Phylogenetic analysis

For DNA extraction, cells from 5 mL of an exponentially grown culture were harvested by centrifugation (3,220 g, 10 min). Then, DNA was isolated using the XS-buffer (xanthogenate-SDS) method as described (Tillett and Neilan 2000). The quality and quantity of the isolated DNA were measured using a NanoDrop™ One device (Thermo Fisher Scientific). Next, polymerase chain reaction (PCR) was carried out using specific primer pairs for two target genes: Amplification of the partial 16S rRNA gene sequences was carried out with the archaeal forward primer 8aF (Burggraf et al. 1992) and the universal reverse primer 1512uR (Lane 1991), while the *mcrA* gene sequence was amplified with MR1 (Simankova et al. 2003) as forward and ME2 (Sakai et al. 2008) as reverse primer.

PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega GmbH, Mannheim) according to the manufacturer's instructions. For bidirectional Sanger sequencing, samples were sent to LGC (LGC Genomics GmbH, Berlin, Germany). Upon receipt of the raw data, sequences were manually revised in 4Peaks 1.7 (Griekspoor and Groothuis 2005) and compared to the BLAST nucleotide database (Altschul et al. 1990) for initial genetic characterization. Alignments were constructed with reference sequences of all validly published *Methanocella* species, as well as with the type strains of phylogenetically neighboring genera. *Methanospirillum hungatei* was used as an outgroup in both cases. Corrected sequences were then aligned using the ClustalW algorithm (Thompson et al. 1994, 2003) in MEGA X (Kumar et al. 2018). Approximately maximum-likelihood trees were calculated with the merged sequences in FastTree 2 (Price et al. 2010) and further visualized and edited using iTol (Letunic and Bork 2007).

Determination of G+C content was done by genome sequencing on an Oxford Nanopore Technologies MinION MK1C sequencer (ONT, Oxford, the United Kingdom; MinKNOW v. 20.10.6). Prior to that, library preparation was achieved in accordance with ONT guidelines for native barcoding of genomic DNA (with EXP-NBD104 and SQK- LSK108). Guppy (fast option, qscore cutoff 7, v. 4.2.3) was used for base calling and demultiplexing. Genome assembly was executed using flye (v. 2.8.2) (Kolmogorov et al. 2019), and finally, the G+C content was calculated from contig sequences in R using Biostrings (Pagès et al. 2022).

Physiological characterization

All physiological experiments were carried out in triplicates. For pH and sodium chloride optima as well as for the utilization test of various substrates, three subsequent transfers were made, if the first inoculation provoked growth. To evaluate the overall growth temperature range, the isolate was inoculated at 15°C, 20°C, 30°C, 35°C, 37°C, 40°C, 42°C, 45°C and 50°C for at least two weeks. The incubations at low temperatures (15–20 °C) were carried out for four weeks in total. Growth was further tested for range and optimal pH, at values from pH 4.0–7.5 in steps of 0.5. The tested concentrations for sodium chloride were 0–5%. Here, the range from 0–1% was tested in steps of 0.2%, while the upper range from 1–5% was evaluated in steps of 0.5%. Substrate utilization for methanogenesis and growth was tested using the following substrates: H_2/CO_2 (80:20, v/v), acetate (17 mM),

formate (22 mM), methanol (31 mM), ethanol (21 mM), 1-propanol (17 mM), 2-propanol (17 mM), 1-butanol (14 mM), 2-butanol (14 mM), methylamine (32 mM), and trimethylamine (17 mM). In addition, a combination of 0.1% acetate and 0.1% yeast extract was tested. Methane production was confirmed by gas chromatography (Agilent 7890A, Agilent Technologies, Santa Clara, USA). For the determination of optimal growth and the calculation of the isolate's doubling times, triplicates were inoculated in SMS medium with H_2/CO_2 and 0.1% acetate at 35°C, 37°C and 40°C. Cell numbers were determined using a Thoma counting chamber (depth: 0.02 mm) every 24 h for a duration of five days until the stationary phase was reached and the generation time was calculated from the optimal growth data at 37°C.

Results and discussion

Enrichment and isolation

In the original biotope, a temperature of 30°C and a pH of 6.5 was measured, which set the starting point for the enrichment and further physiological tests. SMS medium supplemented with 0.1% acetate and H_2/CO_2 gas phase was inoculated with the sample at 37°C for one week until the first rod-shaped, fluorescent cells were microscopically visible. Two subsequent dilution series on the same medium led to the successful isolation of strain CcM2M^T.

Phylogenetic analysis

Phylogenetic comparison of strain CcM2M^T was carried out on 16S rRNA gene sequence and on *mcrA* gene sequence level.

For the 16S rRNA gene sequence, a fragment of 1281 bp was amplified and its sequence was compared with sequences in the NCBI Genbank database using BLASTn. It resulted in 94.43% sequence identity to the family *Methanocellaceae*. As displayed in the phylogenetic tree (**Figure 1**), strain CcM2M^T is found close to the validly described species *Methanocella arvoryzae* (Sakai et al. 2010), *Methanocella paludicola* (Sakai et al. 2008), and *Methanocella conradii* (Lü and Lu 2012) of the genus *Methanocella* and to *Methanooceanicella nereidis*, the only described species within the *Methanooceanicella*. The closest relatives on 16S rRNA level were *Methanocella arvoryzae* with 94.43% and *Methanooceanicella nereidis* with 93.79% gene sequence identity to strain CcM2M^T. No significant other closely related genera could be identified on the 16S rRNA gene sequence level.

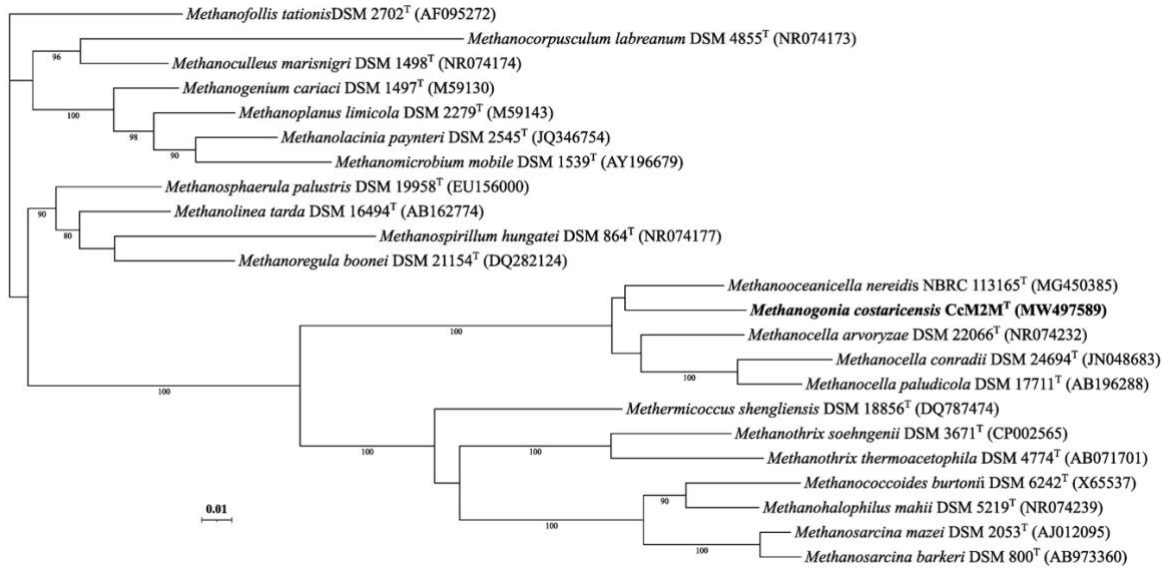

Figure 1. Phylogenetic position of strain CcM2M^T based on 16S rRNA gene sequence of all published *Methanocella* and *Methanooceanicella* species and selected type species of neighboring genera. Bootstrap values greater than 80% are displayed. Bar, 1 substitution per 100 nucleotide positions.

A fragment length of 762 bp was achieved for the *mcrA* gene sequence with a sequence identity of 78.54% as compared to the *mcrA* gene from *M. arvoryzae*. *M. nereidis* was genetically very different on *mcrA* gene sequence

level, and grouped close to *Methanothrix* and *Methanolacina*. The corresponding phylogenetic tree in **Figure 2** shows that isolate CcM2M^T aligned in between the *Methanocella* (Sakai et al. 2008) and *Methanosarcinaceae*, represented by *Methanohalophilus mahii* (Paterek and Smith 1988), *Methanosarcina mazei* (Mah and Kuhn 1984) and *Methanosarcina barkeri* (Bryant and Boone 1987). *Methanothrix soehngenii* (Huser et al. 1982) (formerly *Methanothrix concilii* (Patel 1984) and *Methanosaeta concilii* (Patel and Sprott 1990)) and *Methermicoccis shengliensis* (Cheng et al. 2007) also grouped closely to isolate CcM2M^T. As determined for the 16S rRNA gene sequence analysis, *M. arvoryzae* was also the closest related species on *mcrA* gene sequence level, closely followed by *M. paludicola*, and *M. conradii*. The G+C content of isolate CcM2M^T was 41.0 mol% and thereby significantly lower than all other *Methanocella* species with a G+C content of 52.7–54.9 mol% and different from *Methanooceanicella* with 46.2 mol%. The results of both phylogenetic analyses indicate that strain CcM2M^T closest validly described relative belongs to the family *Methanocellaceae*. Nonetheless, the significant phylogenetic distances of 5.57% and 21.43% on 16S rRNA and *mcrA* gene sequence levels, respectively, justify the assignment to a novel genus.

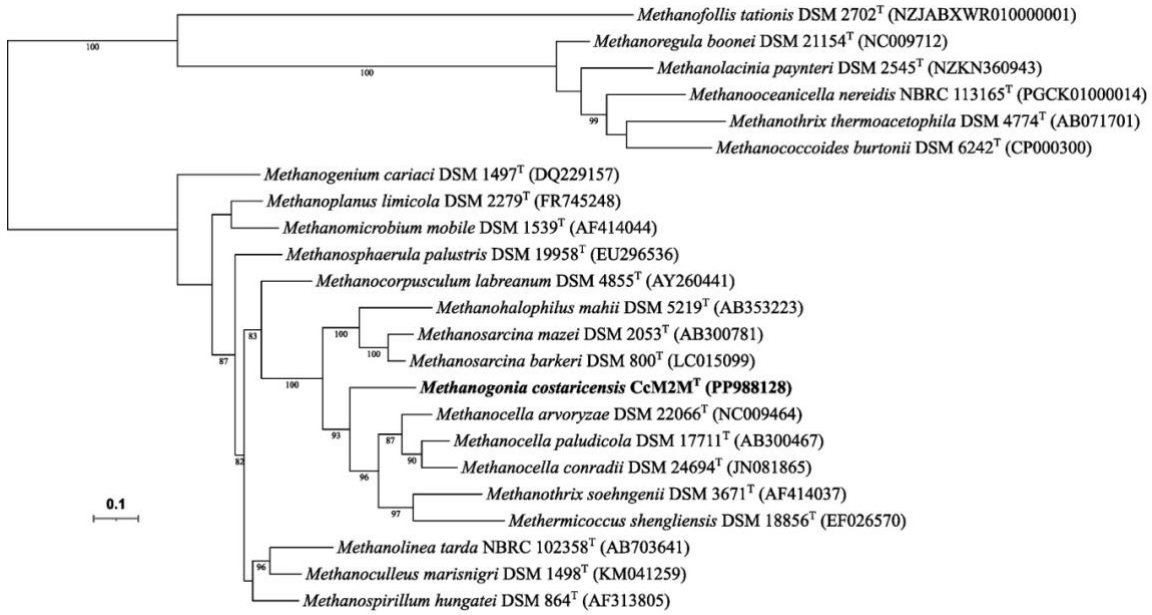

Figure 2. Phylogenetic position of strain CcM2M^T based on *mcrA* gene sequence of all published *Methanocella* and *Methanooceanicella* species and selected type species of neighboring genera. Bootstrap values greater than 80% are displayed. Bar, 10 substitutions per 100 nucleotide positions.

Structural and physiological characterization

CcM2M^T appeared as individual, rod-shaped cells with a diameter of 0.4–0.7 µm and a length of 1.7–2.5 µm and showed the typical methanogenic autofluorescence caused by factor F_{420}. Additionally, some cells showed rather kinked than crooked morphology and always revealed prominent, sharp-edged cell ends (**Figure 3a, Figure 4**). These angular ends were accompanied by a strong electron density at the terminal ends of most cells, as seen in transmission electron micrographs (**Figure 3b**); the appearance is very much in line with the images obtained by phase contrast light microscopy (**Figure 3c**). Using phase contrast light microscopy, the cells of this strain appear with rather undefined outlines. This observation is presumably caused by the irregular cell surface and the crooked nature of the cells. Transmission electron micrographs of Pt/C shadowed cells (**Figure *3***a, b) further indicated

that the ends are very stable (see the shadow casts of the cells), even after air-drying, while for some cells, the body appeared collapsed (as typical for most prokaryotic cells under these conditions).

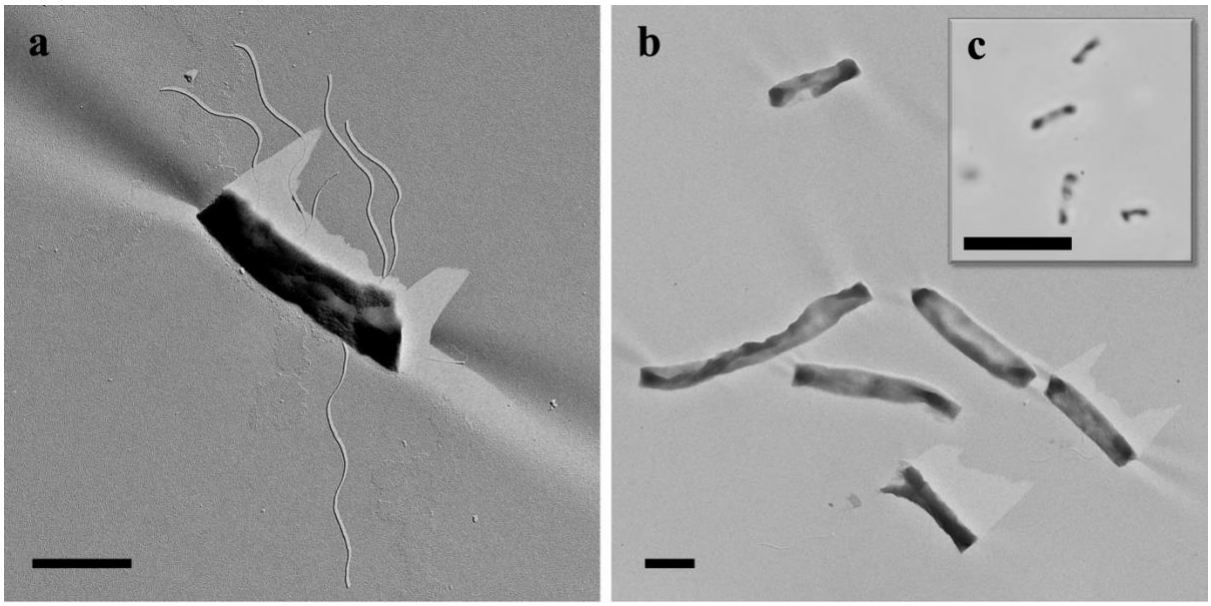

Figure 3. (a, b) Transmission electron micrographs of Pt/C shadowed CcM2M^T cells indicating (a) characteristic, angular and thickened cell ends as well as peritrichous archaella. (b) Thickened structures at the cell ends occur more electron-dense and are again highlighted by long Pt/C shadows. (**a, b)** Bars, 1 µm. (**c**) Phase contrast light microscopic image of isolate CcM2M^T confirms these prominent cell ends and shows partially translucent cell centers. (**c)** Bar, 10 µm.

Freeze-etching highlighted ultrastructural details: the outermost sheath is a proteinaceous S-layer structure with p6 symmetry (**Figure 4**). The total cell envelope, including S-layer and membrane, was about 20 nm in thickness. Additionally, the electron micrograph displayed in **Figure 4** shows one almost complete cell, linked to a second cell (bottom right), plus the outer almost rectangular edges of a third cell (top right; links between the cells highlighted by black triangles). Whether this is indicative of a unique stage of cell division which was captured by the fast-freezing process during freeze-etching, remains to be seen in future studies.

The authors are aware that using electron microscopy only provides a snapshot of the full cell division process. In principle, freeze-etching can reveal fine structural details confidentially, as this method avoids two artifact-prone steps in sample preparation: there is no chemical fixation step, and no dehydration involved. Our finding might therefore be indicative for an extraordinary function of the highly unusual cell ends.

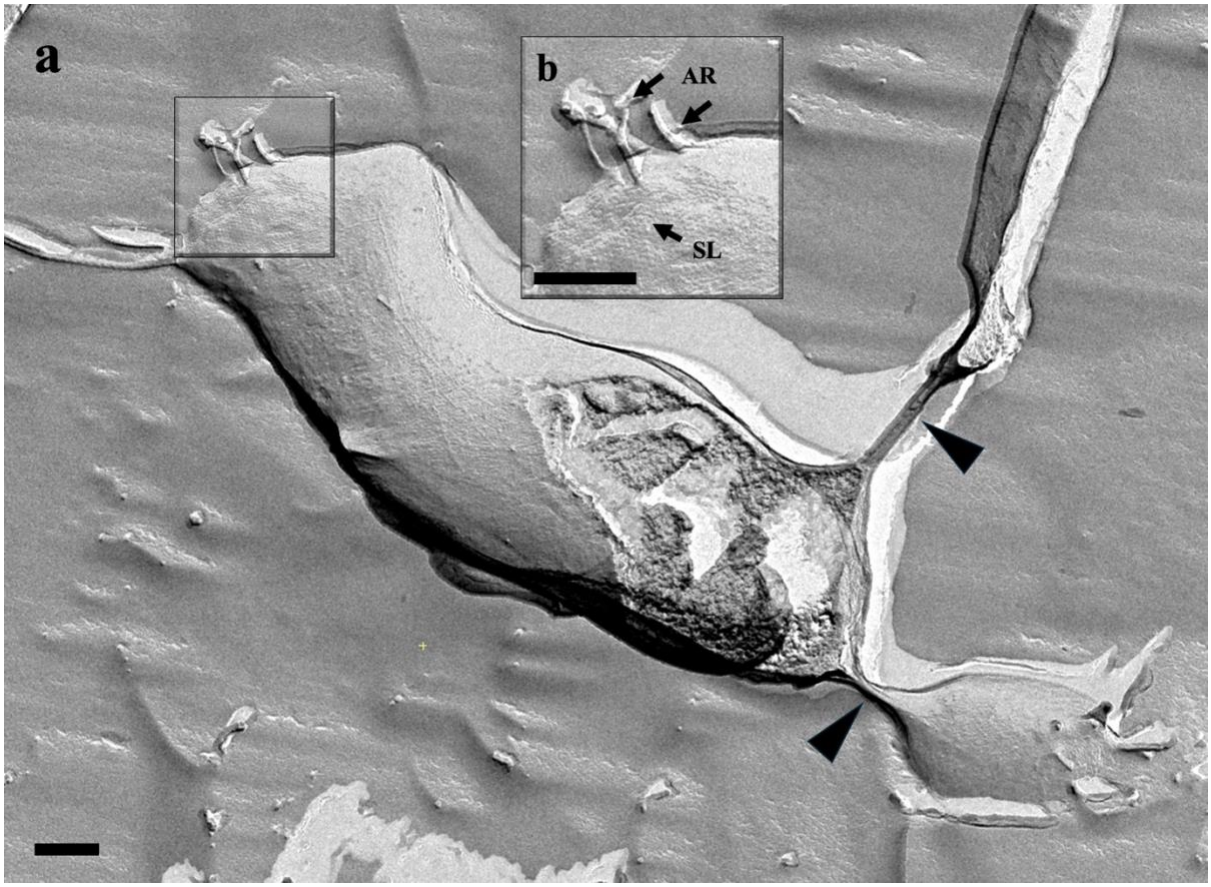

Figure 4. Transmission electron micrograph of a freeze-etched CcM2M^T cell indicating (a) an irregularly shaped rod and kinked cell, as well as (b) archaella (AR) and an S-layer (SL) with p6 symmetry. Black triangles (a) further indicate cell-cell connections, possibly in a late stage of cell division at the edges of the angular cell ends. Bars, 200 nm.

Even though up to 8 peritrichous archaella with a diameter of 12–14 nm were identified (**Figure 3a**), no motility was observed in the temperature gradient-forming device or during other microscopic surveys. In the natural habitat, the cell appendages might therefore be used for attachment rather than for motion, similar as described e.g. for *Pyrococcus furiosus* (Näther et al. 2006), *Methanocaldococcus villosus* (Bellack et al. 2011), and *Ignicoccus hospitalis* (Meyer et al. 2014). Here, they may be used for attachment within the sediment to protect cells from being washed into the ocean. The organism's exceptional cell division strategy might be another conceivable key to keeping individual cells on-site, by forming a three-dimensional, net-like structure with five or more cells. Novel and advanced microscopy studies, which are beyond the scope of this report, will help to unravel the mechanisms involved, including live microscopy of fluorescently labeled cells (Cezanne et al. 2023), and cryo-electron tomography (Rodrigues-Oliveira et al. 2023).

For *M. arvoryzae* and *M. paludicola*, a switch from rod-shaped to coccoid cells was reported in late exponential phase (Sakai et al. 2008, 2010). The authors cannot confirm to have observed exactly the same phenomenon, however, all rod-shaped CcM2M^T cells transformed into cocci after approximately 5 months of storage at 4°C.

CcM2M^T showed a particularly defined temperature optimum with excellent growth at 37°C, significantly weaker growth at 35 and 40°C, and only poor growth at 20–30°C. No growth was observed below 20°C or above 40°C. The optimal pH equaled the pH range and was determined to be pH 5.0–6.5. The strain possessed a wide sodium chloride tolerance and grew equally well from 0–3% NaCl. No growth was observed at sodium chloride

concentrations above 3% and pH levels below 5.0 or above 6.5. The doubling time was calculated to be ~10 h and determined by generating growth curves under optimal growth conditions. Growth and methane production only occurred on H_2/CO_2 (80:20, v/v) with 0.1% acetate as a carbon source. Strain CcM2M^T showed no growth on acetate, formate, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, methylamine, trimethylamine alone or with acetate supplementation. Acetate was required for growth on H_2/CO_2, while yeast extract was not essential, but supported growth. **Table 1** summarizes the unique characteristics of strain CcM2M^T, and the following reference strains were selected for detailed comparison: *Methanocella paludicula* SANAE^T, *Methanocella arvoryzae* MRE50^T, and *Methanocella conradii* HZ254^T display all validly described species of the genus *Methanocella* (Sakai et al. 2008) and represent the most closely related strains to isolate CcM2M^T. The neighboring genera *Methanooceanicella*, *Methanosarcina*, *Methanococcoides*, *Methanothrix*, and *Methermicoccus* are represented by their type species *Methanooceanicella nereidis* CWC-04^T, *Methanosarcina barkeri* MS^T (Mah and Kuhn 1984; Bryant and Boone 1987), *Methanococcoides methylutens* TMA-10^T (Sowers and Ferry 1983), *Methanothrix soehngenii* GP6^T (Huser et al. 1982), and *Methermicoccus shengliensis* ZC-1^T (Cheng et al. 2007).

Isolate CcM2M^T derives from the anoxic sediment of a shallow, mangrove-like river estuary in Cahuita National Park, Costa Rica. This habitat underlies the typical temporal fluctuations of tropical and subtropical habitats including dry and rainy seasons. The surveyed region in Cahuita belongs to the wet climatic group with relatively constant temperatures, and high rain and evaporation rates (Quesada-Román and Pérez-Briceño 2019). These varying water levels combined with the seawater influx from the Caribbean Sea explain the isolate's broad tolerance for sodium chloride. Lastly, the slightly acidic pH optimum along with the acetate dependency hints at the presence of organic acids, derived from the degradation processes of organic matter. Acidophilic methanogens related to RC-I were also enriched from a *Sphagnum* peat bog (Sizova et al. 2003).

This comparative survey illustrates that strain CcM2M^T not only possesses distinct genetic properties but also numerous morphological and physiological characteristics that clearly helps to distinguish it from related genera. With that, we conclude that CcM2M^T represents the type species of a new, third genus within the *Methanocellaceae*, and the globally distributed, but widely uncultured methanogens within the Rice Cluster I.

Taxonomic conclusion

Based on phylogenetic, morphological, and physiological characteristics, strain CcM2M^T is considered to display the type strain of the genus *Methanogonia* gen. nov. (Table 1).

Description of *Methanogonia* gen. nov.

Methanogonia (Me.tha.no.go.ni'a M.L. n. *methanum* methane; Gr. fem. n. *gónia*, knee (techn.), referring to the angularly shaped cell ends; N.L. fem. n. *Methanogonia* a methane-producing Archaeon with rectangular cell ends).

Rod-shaped, non-motile cells with typical angular ends. Stains Gram-negative. Mesophilic. Produces methane from H_2/CO_2. Requires acetate as carbon source. The type species is *Methanogonia costaricensis* sp. nov.

Description of *Methanogonia costaricensis* gen. nov. sp. nov.

Methanogonia costaricensis sp. nov. (cos.ta.ri.cen'sis. L. suf. *-ense -ensis* suffix pertaining to / originating from; M.L. neut. *costaricensis* originating from Costa Rica).

Cells are rod-shaped, with angular cell ends, sometimes kinked, with a diameter of 0.4–0.7 µm and a length of 1.7–2.5 µm. Occurring as single cells. Stain Gram-negative. Non-motile with up to 8 peritrichous archaella, and S-layer with p6 symmetry. Strictly anaerobic. Temperature range for growth is 20°C–40°C (optimum: 37°C). Range and optima for pH 5.0–6.5, and sodium chloride 0–3%. H_2/CO_2 used for methanogenesis, acetate required as carbon source. Doubling time 10 h. Closely related to members of the *Methanocellaceae* with 94.43% sequence identity on 16S rRNA (*Methanocellaceae*) and 78.54% (*Methanocella. arvoryzae*) on *mcrA* gene sequence level. G+C content is 41.0 mol%.

The type strain is CcM2M^T (= DSM 113456^T = JCM 39175^T), isolated from brackish sediment of a mangrove-like river estuary in the Cahuita National Park, Costa Rica.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the *mcrA* gene sequence of strain CcM2M^T are MW497589 and PP988128, respectively.

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Declarations

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Contributions

LD, DG, RR, and HH designed the study. ZEA and RR performed electron microscopy. AB and ZEA executed motility assays. ZEA and JM performed physiological and phylogenetic experiments and created the table. LD collected sampling permits, conducted the sampling, realized phylogenetic tree construction, and wrote the manuscript. RR, HH, and LD evaluated results and created figures. All authors commented on the manuscript.

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Supplementary Material

Sampling location close to the river estuary of Río Suárez in Cahuita National Park, Costa Rica. The freshwater stream enters the Caribbean Sea on the left (river outfall not shown).

Tables

Table 1. Comparative data of strains **1**, CcM2M^T (data from this study); **2**, *Methanocella paludicula* SANAE^T (Sakai et al., 2008); **3**, *Methanocella a*rvoryzae MRE50^T (Sakai et al., 2010); **4**, *Methanocella conradii* HZ254^T (Lü & Lu, 2012); **5**, *Methanooceanicella nereidis* CWC-04^T (Zhang et al, 2024); **6**, *Methanosarcina barkeri* MS^T (Mah & Kuhn, 1984; Bryant & Boone, 1987); **7**, *Methanococcoides methylutens* TMA-10^T (Sower & Ferry, 1983); **8**, *Methanothrix soehngenii* GP6^T (Huser et al, 1982); **9**, *Methermicoccus shengliensis* ZC-1^T (Cheng et al, 2007).

Characteristic	**CcM2M^T**	***Methanocella***			***Methanooceanicella***	***Methanosarcina***	***Methanococcoides***	***Methanothrix***	***Methermicoccus***
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Cell morphology	Rod, angular ends	Rod, coccoid	Rod, coccoid	Rod	Rod	Coccoid spheres	Irregularly coccoid	Rod	Coccoid
Cell diameter (µm)	0.4–0.7	0.3–0.6	0.4–0.7	0.2–0.3	0.5–0.6	1.5–2.0	1.0–2.0	0.8	0.7–1.0
Cell length (µm)	1.7–2.5	1.8–2.4	1.3–2.8	1.4–2.8	1.2–2.9	N.A.	N.A.	2.5–6.0	N.A.
G+C content (mol%)	41.0	54.9	54.6	52.7	46.2	48.8–43.5	42.0	49.0	56.0
Temperature (°C) optimum (range)	37 (20–40)	35–37 (25–40)	45 (37–55)	55 (37–60)	42 (20–45)	30–40 (25–55)	30–35 (<15–<40)	35–40 (10–45)	65 (50–70)
pH optimum (range)	5.0–6.5 (5.0–6.5)	7.0 (6.5–7.8)	7.0 (6.0–7.8)	6.8 (6.4–7.2)	5.35 (5.35–7.31)	7.0 (5.5–7.0)	7.0–7.5 (6.0–8.0)	7.1–7.5 (6.6–7.8)	6.0–6.5 (5.5–8.0)
NaCl (g l^{-1}) optimum (range)	0–30 (0–30)	0 (0–1)	0–2 (0–20)	0–1 (0–5)	10 (0–40)	ND	23 (1–64)	ND	5.5–30 (5.5–64)
Generation Time (h)	10	100.8	8.0	6.5–7.8	ND	ND	5.2	65	5
Substrates for Methanogenesis									
H_2/CO_2	+	+	+	+	+	+	–	–	–
Formate	–	+	+	–	+	–	–	–	–

Acetate	–	–	–	–	–	+	–	+	–
Methanol	–	–	–	–	–	+	+	–	+
Growth requirement									
Acetate	+ r	+ r	+ r	+ r	+ r	–	–	+ r	ND
Yeast Extract	+ s	+ s	+ s	+ s	+ s	+ s	+ s	– i	ND

+, positive; –, negative;

ND, not determined; N.A. not applicable;

s, stimulates growth; r, required; i, inhibits growth.